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Original Research

Keratinolytic and Non-Keratinolytic Fungi in Sewage Sludge

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Abstract

Sewage sludge is being used for reclamation of devastated areas and for fertilization of arable soils. However, sludges contain many harmful components, including pathogenic organisms. Many keratinolytic and associated non-keratinolytic fungi are opportunistic pathogens. Our knowledge on fungal occurrence in sludges and sludge-amended soils and on the health risk posed by the fungi is still not sufficient. The present work was part of extensive studies on actidione-resistant fungal pathogens in sludges and sludge-amended soils. Sludges from the Siemianowice-Centrum wastewater treatment plant, Upper Silesia, Poland were examined. Results obtained by means of three methods, i.e. the dilution pour plating method, hair baiting method and most probable number method were compared. The MPN method combines the dilution and hair baiting methods. The dilution pour plating method was found not to be highly informative as to the occurrence of keratinolytic fungi in sludges, while using the method more information was obtained on non-keratinolytic fungi in the sludge environment. Subsequently, the hair baiting method provided the data on fungal growth in the hair spread over the sludge blanket. This qualitative method has often been used for semiquantitative purposes but does not allow for determining fungal quantities. Such quantities were obtained using the MPN method. The method complemented the results obtained using two other methods. The hair baiting and MPN methods use hair and natural media (sterile sludge, sand, and clay) for examination of sludge fungi. The selectiveness of the MPN method was even higher than that of the hair baiting method. Ecological and epidemiological significance of the MPN results was discussed.

Keywords: keratinolytic and non-keratinolytic fungi, actidione-resistant, sewage sludge, dilution pour plating method, hair baiting method, MPN method

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Introduction

The abundance of keratinous debris of human and animal origin, mainly hairs and keratinized cells of the epidermis, characterizes sewage sludge. Keratinolytic fungi degrade keratin, being the main component of these substrates, while non-keratinolytic fungi accompany keratinolytic fungi, utilizing non-protein components of the substrata and/or the products of keratin degradation [1-2]. It is not surprising, therefore, that the fungi occur in abundance in sewage sludge. Keratinolytic and associated non-keratinolytic fungi should be considered potentially pathogenic to humans and animals [3]. Indeed, the fungi have recorded the agents responsible for mycoses [4] and mycotoxin producers [5]. Studies of the sludge pathogenic fungi are, therefore, of epidemiological significance.

In most studies keratinolytic and non-keratinolytic fungi have been examined with the aid of the dilution pour plating method, using solid media supplemented with actidione and antibacterial antibiotics, and of the hair baiting method [1, 6-15]. Using the dilution pour plating method, more data have been obtained on the occurrence of non-keratinolytic fungi in sludges. Subsequently, the hair baiting method is basically qualitative but it has been often successfully used for semiquantitative purposes. However, this method does not allow determining fungal quantities. Such quantities can be obtained using the MPN method. A series of experiments to compare quantitative and qualitative compositions of sludge fungal pathogens has been performed. The results obtained for the sludge from the Bytom-Miechowice wastewater treatment plant were shown in [16]. The objective of this paper was to compare results obtained with the above-mentioned methods for sludges from the Siemianowice-Centrum wastewater treatment plant and another plant in Upper Silesia, Poland.

Material and Methods

The sludge from the Siemianowice-Centrum wastewater treatment plant, Upper Silesia, Poland, was used in the experiment. It was the excess sludge after extended aeration (without primary settling tank), after integrated biological process for P, N and C removal, and stabilized in a sludge anaerobic digestion chamber. Two sludges were collected. One sludge was dewatered in beltpress and the other was dewatered in beltpress and by gravity in a sludge-drying bed for 6 months. The sludges were examined for selected physico-chemical and bacteriological parameters (Table 5). The methods of sludge sampling and physico-chemical and biological analyses were described in previous papers [1, 9]. The sludges were being dried in open air for 7 days to be used in the experiment.

The following methods were used for examination of sludge actidione-resistant fungi:

(1) dilution pour plating method;
(2) hair baiting method [17] modified by Ulfig [18]; and
(3) MPN method. The MPN method combines dilution and hair baiting techniques.

Sludge dilutions (1:10-1:100,000) were prepared in physiological saline. Total fungal numbers and the numbers of mesophilic and thermophilic fungi were determined using MEA and DG18 at 25ºC (with a 7-day incubation) and YpSs at 37 and 45ºC (with a 4-day incubation), respectively. All media were supplemented with chloramphenicol (100 mg/L). Five plates were set up for each medium, dilution and temperature. The terms "mesophilic" and "thermophilic" were used in the sense of incubation temperature.

Wiegand medium (Bacteriological peptone – 10 g; glucose – 40 g; Bacteriological agar – 20 g; chloramphenicol – 100 mg; actidione – 500 mg; 0.5-% alkaline phenol red solution – 40 mL; redistilled water – 960 mL; pH – 5.6) was used for examination of actidione-resistant fungi [19]. Five plates were set up for each dilution and temperature. The plates were incubated for 10-14 days at 23 and 37ºC. Proteolytic strains change the color of the medium from yellowish to red, due to the pH increase caused by peptone ammonification. The detection limit of the method was 200 CFU/100 g d.w. (dry weight).

In the hair baiting method, a 30 g sludge portion was placed in each plate and watered with sterile redistilled water to obtain ca. 30% moisture. A 0.4 g portion of fine cut, detergent-defatted, autoclaved children's hair was spread over the sludge in each plate. The plates were then incubated at 23, 29, 33 and 37ºC for 4 months. Ten plates were set up for each temperature.

In the MPN method, 10 g portions of sand, clay or sludge were placed in plates and hair (0.4 g) was spread over the medium in each plate. The plates were then autoclaved three times (30 minutes at 121ºC) at 24-hour intervals. Sludge dilutions (1:10 to 1:1,000,000) were prepared with physiological saline. Inoculation was performed by adding a 10-ml dilution portion to each plate (with no mixing). Ten plates were set up for each dilution, medium and incubation temperature. Sterile redistilled water was added to all plates to obtain ca. 30% and 20% moisture for sludge, clay, and sand. The plates were incubated for 4 months at 23, 29, 33 and 37ºC.

During incubation of the hair baiting plates the moisture was kept stable by adding sterile redistilled water.

After each monthly sampling, the hair cuts were examined by microscopy and also spread over plates of Sabouraud 1:10/mineral salts agar (TK medium) [20], supplemented with chloramphenicol (100 mg/L) and actidione (500 mg/L). After incubation at 23 and 37ºC for 10-14 days, growth of a particular species was taken as confirmation of the previous observation by microscopy of that species occurring in the hair.

Fungal isolates from Wiegand medium and hair baiting plates were purified and identified to species level using selected taxonomic monographs [4, 21-27]. The *in vitro* hair degradation test was that of Ulfig et al. [28].

In an MPN combination a single positive or negative result in a repetition derived from observations of four plates incubated at 23, 29, 33 and 37ºC. If a given species appeared at least once in any of the four plates, it was taken as a positive result for the repetition in the MPN combination. MPN counts were calculated following standard rules

Table 1. Fungal quantities in sludges from the Siemianowice-Centrum wastewater treatment plant. Data obtained with the dilution pour plating method.

Parameter	Medium	Temperature	Fungal number [CFU/100 g d.w.] in sludges	
			Dewatered in beltpress	Dewatered in beltpress and by gravity in sludge drying bed
Total fungal number	MEA*	25ºC	5.1×10^5	1.9×10^6
Total fungal number	DG18*	25ºC	3.2×10^5	1.6×10^6
Number of mesophilic fungi	YpSs*	37ºC	2.2×10^5	4.1×10^5
Number of thermophilic fungi	YpSs*	45ºC	<500	<500
Total number of actidione-resistant fungi	Wiegand**	25ºC	5.7×10^4	8.6×10^4
Number of mesophilic actidione-resistant fungi	Wiegand**	37ºC	7.2×10^3	4.1×10^4

* – chloramphenicol (100 mg/L) was added to the medium;
** – chloramphenicol (100 mg/L) and actidione (500 mg/L) were added to the medium.

and the formula given by Geldreich [29, 30]. Ten repetitions were used to obtain high precision of MPN calculations. The detection limit was 1 MPN/100 g d.w.

Results

The total fungal numbers on MEA and DG18 and the number of mesophilic fungi on YpSs were 3.7-, 5- and 1.9-times higher in sludge dewatered in beltpress/sludge drying bed than in sludge dewatered only in beltpress, respectively (Table 1). Subsequently, the number of thermophilic fungi on YpSs was low and identical in both sludges. Finally, the number of total actidione-resistant fungi and the number of mesophilic actidione-resistant fungi were 1.5- and 5.7-times higher in sludge dewatered in beltpress/sludge drying bed than in sludge dewatered by beltpress, respectively.

In total, ten actidione-resistant species were isolated from both sludges with the dilution pour plating method (Table 2). Eight and seven species were recorded in sludge dewatered in beltpress/sludge drying bed than in sludge dewatered in beltpress, respectively. *A. kalrai, Geotrichum* sp. and *P. lilacinus* were found to be the predominating species (>10% in relation to the total number of strains) in the sludges. The numbers of the first two species were 4.7- and 7.3-times higher in sludge dewatered in beltpress/sludge drying bed than in sludge dewatered by beltpress, respectively. The numbers of *P. lilacinus* were found to be of the same order of magnitude in both sludges. *A. kalrai* was the only keratinolytic species isolated from sludges on Wiegand medium.

Altogether, eight keratinolytic species were isolated from sludges with the aid of the hair baiting method (Table 3). Eight species were isolated from each sludge. *Chrysosporium* anamorphs of *A. reticulisporus/fulvescens* (teleomorph *A. reticulisporus*), *G. reticulatus*, *S. brevicaulis* and *C. zonatum* were found to be the predominating species (>10%) in the sludges. *Chrysosporium* anamorphs of *A. reticulisporus/fulvescens*, *G. reticulatus* and *C. zonatum* occurred in both sludges with the same number of strains. However, *A. reticulisporus* (teleomorph) was only observed in sludge dewatered in beltpress/sludge drying bed. *S. brevicaulis* along with *Chrysosporium* anamorphs of *A. clathratus* and *A. curreyi* and *A. mutatus* were isolated more frequently from the sludge dewatered in beltpress/sludge drying bed.

Table 2. Qualitative and quantitative compositions of actidione-resistant fungi in sludges from the Siemianowice-Centrum wastewater treatment plant. Data obtained with the dilution pour plating method and Wiegand medium supplemented with actidione.

Actidione-resistant fungal species	Fungal number [CFU/100 g d.w.] in sludges	
	Dewatered by beltpress	Dewatered by beltpress and by gravity in sludge drying bed
Arthrographis kalrai (Tewari & Macpherson) Sigler & Carmichael*	1.5×10^4	7.0×10^4
Geotrichum sp.	5.5×10^3	4.0×10^4
Paecilomyces lilacinus (Thom) Samson	3.3×10^4	1.2×10^4
Trichoderma koningii Oudem.	5.0×10^3	5.0×10^3
Geotrichum candidum Link ex Leman	-	5.5×10^3
Mucor circinelloides van Tiegh.	5.0×10^2	5.0×10^3
Sporothrix sp.	5.0×10^3	-
Candida sp.	1.5×10^3	-
Isaria felina (Pers.) Fries	-	1.0×10^3
Rhizopus oryzae Went & P. Geerlings	5.0×10^2	-

* – keratinolytic species.

Table 3. Qualitative and quantitative compositions of keratinolytic and non-keratinolytic fungi in sludges from the Siemianowice-Centrum wastewater treatment plant. Data obtained with the multi-temperature hair baiting method.

Fungal species	Number of fungal isolates from sludges	
	Dewatered by beltpress	Dewatered by beltpress and by gravity in sludge drying bed
Keratinolytic species		
Chrysosporium anamorph of *Aphanoascus reticulisporus/fulvescens*	20	20
Aphanoascus reticulisporus (Routien) Hubálek	-	12
Gymnoascus reticulatus Zukal	18	16
Scopulariopsis brevicaulis (Saccardo) Bainier	6	19
Chrysosporium zonatum Al-Musallam & Tan	10	10
Chrysosporium an. *Aphanoascus clathratus* Cano & Guarro	3	12
Chrysosporium an. *Arthroderma curreyi* Berkeley	3	10
Amauroascus mutatus (Quelet) Rammeloo	2	10
Chrysosporium keratinophilum D.Frey ex Carmichael	2	2
Non-keratinolytic fungi		
Pseudallescheria boydii complex (Shear) McGinnis et al.	5	30
Fusarium solani complex (Mart.) Saccardo	2	12
Aspergillus versicolor (Vuill.) Tiraboschi	5	4
Scopulariopsis sp.	5	2
Trichosporon mucoides Guého & M.Th. Smith	4	2
Narasimhella sp.	-	6
Aspergillus fumigatus Fres.	5	-
Gliocladium penicillioides Corda	4	-
Mucor circinelloides van Tiegh.	2	1
Cladosporium cladosporioides (Fres.) de Vries	1	1
Phoma sp.	2	-

In total, eleven non-keratinolytic species were isolated from sludges with the aid of the hair baiting method. Ten and eight species were recorded in sludge dewatered in beltpress/sludge drying bed and in sludge dewatered by beltpress, respectively. *P. boydii* and *F. solani* complexes were the predominating species (>10%) in the sludges. The numbers of both species were found to be higher in sludge dewatered in beltpress/sludge drying bed than in sludge dewatered by beltpress.

Altogether, ten keratinolytic species were isolated from sludges by means of the MPN method (Table 4). Seven and nine species were recorded in sludge dewatered in beltpress/sludge drying bed and in sludge dewatered by beltpress, respectively. *A. kalrai, A. reticulisporus* (anamorph + teleomorph) and *S. brevicaulis* were found to be the predominating species in the sludges. The lowest, *A. kalrai* MPN, was observed on the sludge medium. The MPN values of *Aphanoascus reticulisporus* (anamorph + teleomorph) and *S. brevicaulis* were dependent on the specified medium. *Aphanoascus reticulisporus* had the highest MPN values on clay, while its lowest MPN values were observed on sludge. On the contrary, *S. brevicaulis* preferred the last medium; occurring in high quantities. Low MPN values characterized the other keratinolytic fungi in the sludges.

In total, six non-keratinolytic species were isolated from sludges by means of the MPN method. Five species were recorded in each sludge. *Mucor circinelloides, Candida* sp., *P. boydii* complex and *P. lilacinus* were found to be the predominating species in the sludges. *Mucor circinelloides* had high MPN values in both sludges on sand and clay media, but the fungus did not grow on the sludge medium. In both sludges, high MPN values of *Candida* sp. were only noted on sand. The highest *P. boydii* complex MPN was also determined on sand. The fungus occurred with much higher quantity in sludge dewatered in beltpress/sludge drying bed than in sludge dewatered only in beltpress. Finally, the highest *P. lilacinus* MPN was noted in sludge dewatered in beltpress/sludge drying bed while using the sludge medium.

Table 4. MPN values of keratinolytic and non-keratinolytic fungi in sludges from the Siemianowice-Centrum wastewater treatment plant.

Fungal species	Sludge no.	MPN/100 g d.w. in substrate:		
		Sand	Clay	Sludge
Keratinolytic fungi				
Aphanoascus reticulisporus (Routien) Hubálek	1	1.9×10^5	1.9×10^5	95
	2	199	1.0×10^4	166
Arthrographis kalrai (Tewari & Macpher.) Sigler & Carmichael	1	1.9×10^5	9.8×10^4	1.0×10^4
	2	1.9×10^5	1.9×10^5	3.7×10^4
Chrysosporium anamorph of *Arthroderma curreyi* Berkeley	1	<1	<1	<1
	2	<1	<1	9
Chrysosporium anamorph of *Aphanoascus clathratus* Cano & Guarro	1	<1	<1	<1
	2	<1	<1	9
Chrysosporium indicum (Randhawa & Sandhu) Garg	1	<1	199	<1
	2	<1	<1	<1
Chrysosporium keratinophilum D.Frey ex Carmichael	1	598	199	<1
	2	199	<1	180
Chrysosporium zonatum Al.-Musallam & Tan	1	<1	<1	95
	2	<1	199	2
Gymnoascus reticulatus Zukal	1	<1	180	95
	2	<1	<1	376
Scopulariopsis brevicaulis (Sacc.) Bain.	1	199	<1	1.9×10^5
	2	<1	3.1×10^3	1.9×10^5
Trichophyton terrestre complex Durie & Frey	1	<1	<1	<1
	2	<1	<1	5
Non-keratinolytic fungi				
Candida sp.	1	3.1×10^4	<1	2
	2	4.0×10^3	<1	807
Fusarium solani complex (Mart.) Saccardo	1	<1	<1	<1
	2	<1	<1	2
Mucor circinelloides van Tiegh.	1	1.9×10^5	3.7×10^4	<1
	2	9.8×10^4	1.9×10^5	<1
Mycelia sterilia (brown colony)	1	<1	<1	542
	2	<1	<1	<1
Paecilomyces lilacinus (Thom) Samson	1	<1	182	<1
	2	180	<1	1.1×10^3
Pseudallescheria boydii complex (Shear) McGinnis et al.	1	402	<1	95
	2	3.8×10^4	361	26

sludge No. 1 – dewatered in beltpress;
sludge No. 2 – dewatered in beltpress and by gravity in sludge drying bed for 6 months.

Table 5. Physico-chemical and bacteriological characteristics of sludges from the Siemianowice-Centrum wastewater treatment plant.

Parameter	Unit	Sludges	
		Dewatered by beltpress	Dewatered by beltpress and by gravity in sludge drying bed
pH in H_2O	-	5.9	6.8
Organic carbon	% d.w.	27.5	23.1
Total nitrogen	% d.w.	2.5	1.9
C:N ratio	-	11	12.2
Ammonium nitrogen	$N\text{-}NH_4$ % f.w.	0.4	1.1
Nitrite nitrogen	mg $N\text{-}NO_2$/kg d.w.	0.1	0.3
Nitrate nitrogen	mg $N\text{-}NO_3$/kg d.w.	0.1	0.8
Total sulfur	% d.w.	0.5	0.4
C:S ratio	-	55	57.7
Total phosphorus	% d.w.	0.5	0.4
Available phosphorus	mg P_2O_5/100 g d.w.	292	440
Available potassium	mg K_2O/100 g d.w.	556	500
Total coliforms (TC)	MPN/100 g d.w.	620,000	240,000
Fecal coliforms (FC)	MPN/100 g d.w.	2,100	130

d.w. – dry weight; f.w. – fresh weight.

The pH, conductivity and the contents of organic carbon, total nitrogen, ammonium, nitrite, and nitrate nitrogen were all found to be higher in the sludge dewatered in beltpress/sludge drying bed than in sludge dewatered in beltpress (Table 5). The C:N ratio was also higher in the sludge dewatered in beltpress/sludge drying bed. The contents of total sulfur, total phosphorus, available phosphorus and available potassium were found to be of the same order of magnitude in both sludges. The C:S ratios were 55 and 57.7 for the sludge dewatered in beltpress and the sludge dewatered in beltpress/sludge drying bed, respectively. Subsequently, total and fecal coliforms were higher in the sludge dewatered in beltpress than in sludge dewatered in beltpress/sludge drying bed. No *Salmonella* and zooparasite ova (*Ascaris* sp., *Trichuris* sp. and *Toxocara* sp.) were detected in both sludges.

Discussion

The sludge dewatering in sludge drying bed was found to considerably alter the quantitative and qualitative composition of the fungi examined. The process increased the number of fungi from different groups, including actidione-resistant fungi. It has been confirmed that the dilution pour plating method is not highly informative of the alterations in fungal composition resulting from sludge dewatering [16]. More data were obtained by means of the hair baiting method. There is little doubt that the sludge dewatering in beltpress and sludge drying bed stimulated the growth of *S. brevicaulis, Chrysosporium* anamorph of *A. clathratus* and *A. mutatus,* and the sexual reproduction of *A. reticulisporus*. With the aid of this method more strains of *P. boydii* and *F. solani* complexes were isolated from this sludge compared to the sludge only dewatered in beltpress. In addition, the MPN values of *S. brevicaulis, A. kalrai, P. boydii* complex and *P. lilacinus* were found to be higher in the sludge dewatered in beltpress and sludge drying bed than in the sludge only dewatered in beltpress. Quantitatively, our observations agree with results obtained for the sludge dried at open air [31].

The changes in the fungi composition could have resulted from the improvement of the conditions favoring fungal growth, i.e. better aeration, sludge organic matter degradation and structuralization following sludge dewatering. The C:N ratio and low FC number indicated a high degree of the organic matter stabilization process in the sludge dewatered in beltpress/sludge drying bed. However, high MPN values of *S. brevicaulis, C. keratinophilum, A. clathratus, A. reticulisporus* and *A. kalrai* together with a low MPN value of the soil dermatophyte, *T. terrestre* complex, and high ammonium nitrogen concentration testified that the sludge organic matter stabilization process was not yet terminated. Since application of non-stabilized sludge to land may result in serious hygienic problems, e.g. intensive odors, our knowledge on sludge-fungi compositions is significant from the practical point of view. The results have confirmed that the fungi can be used as an indicator of the sludge stabilization process [1, 9, 15].

In some cases MPN values were dependent on the specified medium used for hair baiting and on the sludge sample. For instance, from among keratinolytic fungi *A. reticulisporus* showed the highest quantities in both sludges on clay, and on sand in the sludge dewatered in beltpress. The highest quantities of *S. brevicaulis* were obtained on the sludge medium. *A. kalrai* had high MPN values in both sludges on all media. From among non-keratinolytic fungi *Candida* sp. showed the highest MPN values in both sludges on sand. High *M. circinelloides* MPN values were determined in both sludges on sand and clay, whereas the sludge medium inhibited the growth of this fungus in the hair.

Some keratinolytic species such as *A. kalrai, C. indicum, C. zonatum,* and *T. terrestre* complex were not isolated from sludges by means of the hair baiting method but grew in the hair while using the MPN method. It is believed that the sludge factors inhibited the growth of the fungi but the dilution and sterile media stimulated the organisms to grow in the hair. Interestingly, a similar phenomenon was observed for *Uncinocarpus reesii*, while determining MPN values of keratinolytic and non-keratinolytic fungi in municipal landfill soils [32].

As mentioned earlier, keratinolytic and associated non-keratinolytic fungi are opportunistic organisms; possessing potential pathogenic properties to humans and animals. However, some of the fungi such as *M. gypseum* and *P. boydii* complexes have higher pathogenic potential compared to others [33, 34]. *M. gypseum* belongs to the so-called geophilic dermatophytes; having soil as their main natural reservoir. The dermatophytes invades and multiplies within keratinized tissues (skin, hair, and nails) *in vivo*, causing skin infections. *M. gypseum* was not isolated from the sludges examined, although the fungus were found to occur abundantly in other sludges. It was suggested that the occurrence of *M. gypseum* in sewage sludge depended on C:S ratio (<30) [9]. The present results indicate that the total sulfur content, (>1% d.w.) more than C:S ratio, is the factor responsible for the occurrence of this dermatophyte in sewage sludge.

In the present study the highest *P. boydii* complex quantities was observed on sand in both sludges examined. This finding has confirmed that the fungus favors the sand medium for growth [16]. The actidione-resistant strains of *P. boydii* complex has been found to regularly occur in sewage and sewage sludge [9]. The sludge isolates were molecularly identified (ITS 1 region and *β*-tubulin and calmodulin genes sequencing) as *P. boydii* and *S. auranti-acum* (J. Cano, 2009, personal communication). The fungi from the genus *Scedosporium* have also been recorded in bioaerosols [35, 36] but no data on the aerozolization of their spores from sewage and sludge surfaces have been found in the available literature.

P. boydii (anamorph *Scedosporium boydii*) is a significant opportunistic ascomycete (Microascales) with a high level of resistance to antifungal agents. *P. boydii* was previously known to be involved in subcutaneous infections and in asymptomatic pulmonary colonization in immunocompetent patients but the fungus has been recently recorded from severe infections in immunocompromised and immunosuppressed and, occasionally, in immunocompetent patients [37]. *S. aurantiacum* has recently been found to be more virulent than *P. boydii* [38]. It is assumed that the *Scedosporium* spores enter the respiratory tract *via* inhalation and cause lung transient colonization in predisposed patients (*cystic fibrosis,* post-infectious pulmonary cavities, etc.). In immunosuppressed patients the transient colonization may evolve into persistent colonization [39]. Direct contact of eyes and skin with contaminated sludge or sludge-amended soil may also lead to infection. The virulence of the species from the *P. boydii* complex was evaluated in a murine model [38]. In relation to humans minimal infection doses or dose-response relationships have not been yet determined. In the case of immunocompromised or immunosuppressed individuals, however, even small spore amounts may be dangerous to health. By providing spore quantities in sludges, the MPN method could be helpful in assessing health risk resulting from sludge handling and application to land.

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